Analysis and biochemical characterization of degradation products of anti-Alzheimer's drugs

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2011

document version
Publisher's PDF, also known as Version of record

Chapter 6

Summary, conclusion and perspectives

Stability studies form an important stage in drug development processes. This importance lies in the fact that degradation products might show toxic, as well as genotoxic and/or carcinogenic properties. Moreover, degradation might simply diminish drug efficacy. On the other hand, degradation products can also present enhanced properties when compared to the parent drug and might even become a lead candidate. Stability studies are investigated based on guidelines issued by the International Conference on Harmonisation (ICH). These guidelines suggest that stability tests include investigation of the effects of pH, temperature, humidity, irradiation and oxidizing agents.

This thesis presents stability studies of three anti-Alzheimer's drugs: tacrine, huperzine A and galantamine. All studies were based on the ICH guidelines. Degradation products of the three drugs were characterized chemically and biochemically, by elucidating their chemical structure and investigating their bioactivity against the target enzyme acetylcholinesterase (AChE).

In Chapter 1, a general introduction of the thesis was given with a particular focus on Alzheimer's disease. The ICH guidelines for drug testing have been highlighted.

In Chapter 2, a literature review regarding the degradation, metabolism, and bioanalysis of the anti-Alzheimer's drugs used in the past and in the presence was presented (physostigmine, tacrine, donepezil, rivastigmine, galantamine and huperzine A). Degradation studies were reviewed, presenting the degradation products formed under different stress conditions. Additionally, the bioanalysis of these substances was reviewed giving an overview on analytical methodologies used for sample preparation, separation and detection of the drug substances and their metabolites in different biological matrices. Moreover, the metabolism pathways of these drugs were illustrated. The study of drug metabolism is also very important, because on the one hand metabolites might also exhibit genotoxic or carcinogenic properties (Chefson and Auclair, 2006, Rooney *et al.*, 2004), on the other hand, the metabolites produced may be more potent than the parent compound, potentially leading to new drug candidates (Chefson and Auclair, 2006). The literature research showed that stability studies were investigated in bulk substance mainly, but in the case of donepezil an extemporaneously prepared oral liquid was also analyzed. It was shown that liquid chromatography was the most frequently used method to separate the parent compound and its degradation products; this was also true for metabolism studies. Liquid-liquid extraction was the preferred sample treatment used by most of the authors, in some cases solid-phase extraction was also an option. Interestingly, fluorescence could be used for all the studied drug substances, which even proved to be sufficiently sensitive for pharmacokinetics studies in plasma samples.

In Chapter 3, a fluorescence based on-line bioassay was applied to determine the bioactivity of tacrine degradation products. Tacrine showed degradation only under oxidizing conditions (H_2O_2). This sample was analyzed by a bioassay coupled on-line to high-resolution mass spectrometry and ultraviolet detection. Results showed mainly mono-, di-, and trioxygenated compounds, which also show bioactivity against the target AChE. The degradation products generated by H_2O_2 were compared with the metabolites generated by rat and

pig liver CYPs (cytochrome P450's). Mainly, six compounds were found for both pig liver microsomal incubations and chemical degradation, whereas three compounds were generated by both rat liver microsomal incubations and chemical degradation. Not only in liver microsome incubations but also during degradation with H_2O_2, the main products found were formed by the multiple addition of oxygen (Marques *et al.*, 2010). This observation was especially interesting as these compounds could be easily formed in batch amounts, thereby generating sufficient material for NMR analysis. This statement was sustained by fractionating a higher amount batch treated with H_2O_2. Collected fractions gave sufficient material for NMR analysis. For one of the ketone containing degradation products of tacrine, a full structural elucidation could be carried out by NMR.

In Chapter 4, a stability study of the anti-Alzheimer's drug huperzine A is presented. Stability studies under different pH values and in oxidizing environment have been previously reported by Ashani *et al.* (Ashani *et al.*, 1992). However, photolytic degradation has not been reported previously. Thus, the stability of huperzine A in aqueous environment was studied by irradiating the sample with wavelengths above 310 nm. Under these conditions, huperzine A was converted to a photoisomer named photohuperzine A. Interestingly; this compound lost its absorption maximum at 308 nm (typical of 2-pyridone rings). This observation proved to be a good starting point for our attempts to elucidate the structure of the formed photoisomer. Diverse NMR techniques were used for its structural characterization. It was observed, that photocyclization of the 2-pyridone ring had occurred. Curiously, the compound lost its bioactivity against AChE, showing a 100 times lower activity than huperzine A itself. The MM2 energy minimized structures, which showed the loss of planarity of the pyridone ring gave a possible explanation for this finding.

In Chapter 5, the stability study of the anti-Alzheimer's drug galantamine (3-methoxy- 11-methyl- 5,6,9,10,11,12-hexahydro-4aH-[1] benzofuro [3a, 3, 2-ef][2] benzazepin-6-ol) is presented. Degradation kinetics, structure elucidation and bioactivity assessment of degradation compounds was assessed. In acidic environment, an epimerized product was formed (epigalantamine) as well as two dehydrated products, one of which could be characterized as 3-methoxy-11- methyl-9,10,11,12-hexahydro-4aH-benzo[2,3]benzofuro[4,3-cd] azepine. Under high pH and high temperatures, galantamine proved to be stable. Under oxidizing conditions, galantamine showed the formation of a major product characterized as *N*-oxide-galantamine. Under photolytic conditions (> 310 nm), a huge number of isomers is formed, two of which could be characterized as epigalantamine and 3-methoxy-11-methyl-7,8,9,10,11,12-hexahydro-4aH-benzo[2,3]benzofuro[4,3-cd] azepin -6(5H)-one with the same elemental composition as galantamine ($C_{17}H_{21}NO_3$). Degradation kinetics was assessed. First-order kinetics was observed under acidic and photolytic conditions. Under oxidizing conditions, two-phase decay kinetics was observed. This fact is not fully understood as degradation of H_2O_2 was not observed after titration before and after reaction at 80°C for more than 2 hours. Degradation products formed under acidic conditions showed no activity or less activity than the parent drug itself.

To conclude this thesis, an overview of the analytical methodologies used for degradation analysis, bioanalysis and metabolism of the presented anti-Alzheimer's drugs used in the past and at present is presented. A table for the convenient guidance of the reader towards suitable methods for his/her analytical problem can be found in Chapter 2.

The on-line bioactivity analysis of degradation products provided fast information about active degradation products formed during stability assays. Even if the compound is presented in small amounts in the mixture, it can be detected by the bioassay, if the affinity between compound and target is strong (Patrick, 2009). This research showed that chemical degradation can yield similar compounds as biological degradation (CYPs) and also novel bioactive compounds. Chemical degradation (H_2O_2 in the case of tacrine) can thus conveniently be used to generate sufficient amounts of metabolites or other bioactive compounds also found after biological degradation for further analysis, e.g., NMR spectroscopy. Choosing for either on-line or off-line biochemical characterization will depend on the complexity of the matrix and the final goal of the analysis. If the goal is the screening for active compounds in a complex mixture, the on-line biochemical characterization is advisable. If IC50 values have to be determined, the off-line biochemical characterization is more appropriate for a small number of samples; in cases where of a large number of compounds has to be characterized, an on-line biochemical characterization using flow-injection-analysis (FIA) is suggested to reduce time and to reduce errors by repetitive pipeting. Note that for both cases a pure compound is necessary, by means of a commercial standard sample or by means of a pure extract obtained via preparative liquid chromatography.

Moreover, this thesis presented a new, not previously described compound formed by photoisomerization of huperzine A, named photohuperzine A. Although this compound showed no activity against AChE, the fact strengthens the importance to perform stability studies for drug substances. The study also showed the strong relation between the planarity of the 2-pyridone ring in huperzine A and the active site of AChE: when this planarity is lost, no activity is observed. This important observation can help the scientific community to design new drugs, or the new compound could be interesting for the use against other diseases.

Furthermore, this thesis showed a complete stability study for galantamine, including kinetics determination, structure elucidation, and bioactivity analysis. Here, the formation of two degradation compounds (*N*-oxide-galantamine and epigalantamine) was observed, which were also reported as galantamine metabolites in rat liver microsomal incubations (Malakova *et al.*, 2007). As in the case of tacrine, chemical degradation could possibly be used to generate some of the metabolites of galantamine, in order to be used as reference standards.

Some of the concepts presented in this thesis will continue to play a role in the group's research. Namely, the formation of substance mixtures by incorporating for example oxidizing conditions to transform known inhibitors into possible new compounds. At present, this is investigated for kinase inhibitors. Kinases play an important role in inflammatory diseases, which makes these enzymes very important drug targets (Cohen, 2002). In this project, the degradation products

are primarily generated electrochemically, but chemical degradation with H_2O_2 is performed for comparison. The electrochemical setup is coupled on-line with a fluorescence based bioassay to detect for bioactive conversion products of known kinase inhibitors (Falck *et al.*, 2010). Electrochemical degradation by oxidizing the drugs using a glass carbon working electrode was earlier demonstrated by Karst (Karst, 2004). The advantage of this technique is that the degradation can be monitored by simply changing the potential of the electrode (Karst, 2004). It was reported that this method very well mimics phase I metabolism, and also allows the detection of reactive metabolites, which is not possible under in vitro and in vivo conditions (Lohmann and Karst, 2007).

In the near future, most of the separation methodologies for drug stability analysis, bioanalysis and metabolism studies will opt for methods which bring enhanced speed and resolution as main factors. The ultra-high-performance liquid chromatography (UPLC) is an option which shows this characteristic and will probably become the method of choice for future developments. Very recent papers regarding stability studies of β-blockers and diuretic drugs (Narasimham and Barhate, 2010), desloratadine (Rao *et al.*, 2010), sparfloxacin (Gupta *et al.*, 2010) already employed UPLC in their methodology. Capillary electrophoresis (CE) certainly is another technique which also brings enhanced speed and resolution. A review from Altria, published in 1996, describes the use of CE to determine drug-related impurities of pharmaceutical substances (Altria, 1996). Here, the author states that CE methods are capable of validation in this area and can often give equivalent performance to HPLC methods. He adds that the adoption of CE for these tests can be beneficial by reducing costs and improving robustness. This review of 14 years ago was very positive about the use of CE in the field of pharmaceutical analysis. At the present, CE is unquestionably a strong technique to be used for this kind of analysis. This is especially true because over these 14 years various new developments in CE took place. Next to the development of new capillary coatings (Ramautar *et al.*, 2009), several new CE techniques have been introduced (Suntornsuk, 2010). Non-aqueous capillary electrophoresis (NACE) is based on the use of pure or mixed organic solvents as the background electrolyte (BGE). Microemulsion electrokinetic chromatography (MEEKC) is based on chromatographic partition of analytes between microemulsion droplets and the aqueous BGE. Capillary electrochromatography (CEC) is based on chromatographic partition of analytes between stationary phases and the BGE. Immunoaffinity capillary electrophoresis (IACE) involves capturing of a target analyte from a complex mixture onto an immobilized 'affinity ligand' bound to a solid support and subsequent CE separation of the compounds released from the immobilized affinity ligands. In addition different means of detection have been developed (MS, light emitting diode (LED), fluorescence, chemiluminescence (CL), and contactless conductivity (C4D) detectors) (Suntornsuk, 2010).

Several examples of the use of CE in stability-indicating methods have been reported, including methods for norflaxin (Alnajjar *et al.*, 2007), azaphenothiazine (Baalbaki *et al.*, 2005), metformin (Hamdam *et al.*, 2010). Table 6.1 illustrates a comparison between HPLC, UPLC and CE. In summary, both UPLC and CE can be considered important techniques that improve the area of pharmaceutical analysis by speeding up the analysis and reducing analysis cost.

Another area of pharmaceutical analysis where significant developments are to be expected in the near future is the analysis of genotoxic impurities. In the absence of appropriate evidence supporting the existence of a threshold for a genotoxic compound it is difficult to define a safe dose. Therefore, it is necessary to adopt a concept of a level of exposure that carries an acceptable risk. Based on this considerations, genotoxic impurities are distinguished into the following two classes: genotoxic compounds with sufficient (experimental) evidence for a threshold-related mechanism, and genotoxic compounds without sufficient (experimental) evidence for a threshold-related mechanism (EMA, 2006). The European Medicines Agency (EMA) stipulates an acceptable limit of genotoxic impurities in drug substances at value of 1.5 μg/person/day. This value was based on a threshold of toxicological concern, which was defined as a common exposure level for any unstudied chemical that will not pose a risk of significant carcinogenicity or other toxic effects (EMA, 2006). Values of threshold for genotoxic impurities are not (yet) defined in the ICH.

In a review, published in 2010, Kushwaha states: "For genotoxic impurities, we need very sensitive and selective methods. One needs higher selectivity to determine ppm-level impurities and selective methods to separate low levels of genotoxic impurities from base line noise and other organic impurities. The typical HPLC methods with a nonspecific detector (e.g UV) that are used to measure organic impurities may not be appropriate to quantitate low ppm levels of genotoxic impurities. The quantitation of low levels (in the range of ppms) of impurities is the challenging part, and using specific detectors as MS or MS-MS with LC will significantly improve the method selectivity and the quantitation limit. The goal for scientists is to identify potential genotoxic impurities early in development, develop analytical methods to test for these impurities in the intermediate, and if possible, to demonstrate that the manufacturing process controls them before reaching the final drug substance. If you eliminate them early enough, then your actual active drug substance is pure, free of genotoxic impurities" (Kushwaha, 2010). Certainly with the advance of the analytical techniques hopefully the challenge of detecting small levels of genotoxic compounds will be achieved in the near future.

Table 6.1 Comparison of HPLC, UPLC and CE adapted from (Altria, 1996, Nováková *et al.*, 2006).

	HPLC	UPLC	CE
Sensitivity	+ + +	+ + +	+ + +
Precision at low levels	+ + +	+ + +	+ +
Low UV wavelength detection	+	+	+ + +
Indirect UV detection	-	-	+ +
Preparative isolation	+ +	+ +	-
Running costs	+	+ +	+ + +
Sample preparation needs	+ +	+ +	+ +
Main peak assay	+ + +	+ + +	+ +
Automation	+ + +	+ + +	+ +
Robustness	+ +	+ +	+ +
Analysis time (1-5 samples)	+	+	+ +
Analysis time (5-50 samples)	+ +	+ + +	+ +
Column etc. costs	+	+	+ + +
Experience	+ + +	+ + +	+
Selectivity	+ +	+ +	+ +

Key: - poor; + acceptable; + + good; + + + excellent

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Nederlandse samenvatting

Analyse en biochemische karakterisering van afbraakproducten van anti-Alzheimer's medicijnen

Stabiliteitsstudies vormen een belangrijke fase in het ontwikkelingsproces van geneesmiddelen. Dit ligt aan het feit dat de afbraakproducten toxisch, genotoxisch en/of kankerverwekkend kunnen zijn. Bovendien zou degradatie simpelweg de werkzaamheid van het geneesmiddel kunnen verminderen. Aan de andere kant kunnen de afbraakproducten ook verbeterde eigenschappen hebben ten opzichte van het oorspronkelijke geneesmiddel en misschien zelfs een "lead-kandidaat" zijn. Stabiliteitsstudies worden verricht op basis van de richtlijnen die door de Internationale Conferentie voor Harmonisatie (ICH) zijn uitgegeven. Deze richtlijnen suggereren dat de stabiliteitsstudies onderzoek omvatten naar de effecten van de pH, temperatuur, vochtigheid, licht en oxidatieve reagentia.

Dit proefschrift beschrijft de stabiliteitsstudies voor drie anti-Alzheimer geneesmiddelen: tacrine, huperzine A en galantamine. Alle studies zijn gebaseerd op de ICH-richtlijnen. Afbraakproducten van de drie geneesmiddelen werden zowel chemisch als biochemisch gekarakteriseerd, door het ophelderen van hun chemische structuur en het onderzoeken van hun bioactiviteit tegen het beoogde enzym acetylcholinesterase (AChE).

Hoofdstuk 1 geeft een algemene inleiding van het proefschrift met een bijzondere nadruk op de ziekte van Alzheimer. De ICH richtlijnen voor stabiliteitsstudies worden kort besproken.

Hoofdstuk 2 is een literatuuroverzicht van de beschikbare kennis van en methoden voor de bestudering van de afbraak, het metabolisme en de kwantitatieve bioanalyse van de anti-Alzheimer middelen die in het verleden en tot op vandaag gebruikt worden (fysostigmine, tacrine, donepezil, rivastigmine, galantamine en huperzine A). Degradatiestudies en de afbraakproducten gevormd onder verschillende stress-condities worden beschreven. Daarnaast wordt ook de kwantitatieve bioanalyse van deze componenten besproken, waarbij aandacht wordt besteed aan de analytische methoden die worden gebruikt voor de monstervoorbereiding, scheiding en detectie van de geneesmiddelen en hun metabolieten in verschillende biologische matrices. Bovendien wordt het metabolisme van deze geneesmiddelen geïllustreerd. De metabolismestudie is ook belangrijk, omdat enerzijds de metabolieten ook genotoxische of carcinogene eigenschappen kunnen vertonen, en er anderzijds metabolieten geproduceerd kunnen worden die hogere activiteit hebben dan de oorspronkelijke verbinding, wat mogelijk kan leiden tot nieuwe kandidaat-geneesmiddelen. Uit het literatuuronderzoek is gebleken dat in de stabiliteitsstudies vooral de bulkstof werd onderzocht, maar in het geval van donepezil werd eveneens een ex-tempore orale vloeistof geanalyseerd. Vloeistofchromatografie is de meest gebruikte methode om de verbinding, haar afbraakproducten en/of metabolieten te analyseren. Vloeistof-vloeistof extractie heeft de voorkeur van de meeste auteurs voor de monstervoorbehandeling, terwijl in sommige gevallen ook vaste-fase extractie werd toegepast. Ietwat verrassend kan voor alle onderzochte geneeskrachtige stoffen fluorescentiedetectie worden gebruikt,

welke zelfs voldoende gevoelig is voor farmacokinetisch onderzoek in plasma monsters.

In Hoofdstuk 3 werd een op fluorescentie gebaseerde on-line bioassay toegepast om de bioactiviteit van tacrine afbraakproducten te bepalen. Tacrine bleek alleen te ontleden onder oxiderende omstandigheden (H_2O_2). Het monster werd geanalyseerd met behulp van een on-line gekoppelde bioassay met parallel hoge-resolutie massaspectrometrie en ultraviolet-detectie. De resultaten toonden vooral mono-, di-, en tri-geoxideerde verbindingen, die ook bioactiviteit tegen het target AChE vertonen. De H_2O_2 degradatieproducten werden vergeleken met de metabolieten die verkregen werden uit incubatie met ratten- en varkenslevermicrosomen (cytochroom P450's). Zes verbindingen werden voornamelijk gevonden voor zowel incubaties met varkenslevermicrosomen als chemische degradatie, terwijl drie verbindingen werden gegenereerd door zowel incubaties met rattenlevermicrosomen als chemische degradatie. In beide gevallen werden de belangrijkste producten gevormd door de meervoudige toevoeging van zuurstof. Dit kan vooral interessant zijn omdat via H_2O_2 degradatie deze verbindingen gemakkelijk in voldoende mate gevormd kunnen worden voor NMR-analyse. Voor één van de keton-afbraakproducten van tacrine werd voldoende materiaal ingezameld voor een volledige structuuropheldering met behulp van NMR.

In Hoofdstuk 4 wordt een stabiliteitsstudie van het anti-Alzheimer geneesmiddel huperzine A gepresenteerd. Stabiliteitsstudies onder verschillende pH-waarden en in een oxiderende omgeving werden al eerder beschreven door Ashani *et al.*, die echter niet de fotolytische degradatie bestudeerden. Daarom werd de stabiliteit van huperzine A in waterig milieu bestudeerd door het bestralen van het monster met licht met golflengten boven 310 nm. Onder deze omstandigheden werd huperzine A omgezet in een fotoisomeer genaamd photohuperzine A. Interessant was dat deze verbinding het absorptiemaximum bij 308 nm (typerend voor 2-pyridon ringen) verloor. Deze waarneming bleek een goed uitgangspunt voor onze pogingen om de structuur van het gevormde fotoisomer op te helderen. Diverse NMR technieken werden gebruikt voor de structuurkarakterisering. Er werd waargenomen, dat een fotocyclisering van de 2-pyridonring had plaatsgevonden. Vreemd genoeg, verloor de verbinding hierbij grotendeels haar bioactiviteit tegen AChE. De activiteit was 100 keer lager dan huperzine A zelf. De MM2 energie-geminimaliseerde structuren, die het verlies van vlakheid van de pyridonring toonde, gaf een mogelijke verklaring voor deze bevinding.

In Hoofdstuk 5 wordt de stabiliteitsstudie van het anti-Alzheimer geneesmiddel galantamine (3-methoxy-11-methyl-5,6,9,10,11,12-hexahydro-4AH-[1] benzofuro [3a,3,2-EF] [2] benzazepin-6-ol) gepresenteerd. Afbraakkinetiek, structuuropheldering en bioactiviteits-beoordeling van de degradatieproducten werd bestudeerd. In een zure omgeving werd een epimeerproduct (epigalantamine) gevormd en twee gedehydrateerde producten, waarvan er één gekarakteriseerd zou kunnen worden als 3-methoxy-11-methyl-9-,10,11,12 hexahydro-4AH-benzo [2, 3] benzofuro [4,3-cd] azepine. Onder basische condities en hoge temperaturen bleek galantamine stabiel te zijn. Onder oxiderende omstandigheden bleek een belangrijk degradatieproduct van galantamine het *N*-oxide-galantamine te zijn. Onder fotolytische omstandigheden (> 310 nm)

werd een groot aantal isomere producten gevormd met dezelfde elementaire samenstelling als galantamine ($C_{17}H_{21}NO_3$), waarvan er twee kunnen worden gekarakteriseerd als epigalantamine en 3-methoxy-11-methyl-7-,8,9,10,11,12 hexahydro-4AH-benzo [2,3] benzofuro [4,3-cd] azepin -6 (^{5}H)-. Ook de afbraakkinetiek werd bestudeerd. Eerste-orde kinetiek werd waargenomen onder zure en fotolytische condities, terwijl onder oxiderende omstandigheden een tweefase exponentiële kinetiek werd waargenomen. Dit laatste wordt niet volledig begrepen omdat er geen afbraak van H_2O_2 werd waargenomen (via titratie vóór en na de reactie bij 80 °C gedurende meer dan 2 uur). Afbraakproducten gevormd onder zure omstandigheden vertoonden geen of minder activiteit dan galantamine zelf.